Vol. 56: 65–74, 2003 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published August 15

Parasitism by *Dermocystidium ranae* in a population of *Rana esculenta* complex in Central Italy and description of *Amphibiocystidium* n. gen.

R. Pascolini[1], P. Daszak[2,*], A. A. Cunningham[3], S. Tei[1], D. Vagnetti[1], S. Bucci[4], A. Fagotti[1], I. Di Rosa[1]

[1]Department of Cellular and Molecular Biology, University of Perugia, Piazza dell'Universita, 1, 06100 Perugia, Italy
[2]Consortium for Conservation Medicine, 61 Route 9W, Palisades, New York 10964, USA
[3]Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, United Kingdom
[4]Department of Physiology and Biochemistry, University of Pisa, Lungarno Pacinotti, 43, 56124 Pisa, Italy

ABSTRACT: We report the enigmatic parasite *Dermocystidium ranae* in a green frog population (Solomeo, Umbria, Italy) of the *Rana esculenta* complex, consisting of the parental species *R. lessonae* (*L*) and hybrid form *R. esculenta* (*E*). In this population a rapid 50% decline of the parental form *L* was observed. Large dermal U-shaped cysts of *D. ranae* were found primarily on the ventral aspect of infected individuals, with a significantly higher incidence of infection in the parental species compared to the clonal hybrid. In each form, however, there was little pathological change associated with infection, and the cause of the recent declines of *R. lessonae* at this site remains unknown. In this paper we present the first ultrastructural description of an amphibian *Dermocystidium* sp. and we review the taxonomy of *Dermocystidium*, *Dermosporidium* and *Dermomycoides* spp. from amphibians. We conclude that *Dermosporidium multigranulare* Brož & Kulda, 1954 is synonymous with *Dermocystidium ranae* Guyénot & Naville, 1922 and, due to lack of sufficient differences between genera and significant dissimilarities with fish *Dermocystidium* spp., the 3 amphibian genera are synonymous. We propose that they should be designated to a new genus, *Amphibiocystidium* n. gen., and *Dermocystidium* retained for those species parasitic in fish.

KEY WORDS: Amphibian · *Rana* · *Dermocystidium* · *Amphibiocystidium* · Amphibian declines

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INTRODUCTION

In recent years, a global decline in amphibian populations has occurred (Blaustein & Wake 1990, Houlahan et al. 2000). A number of anthropogenic factors have been causally implicated in these declines, including habitat loss, introduction of predators, chemical pollution and climate change (Halliday 2001, Kiesecker et al. 2001). Recently, a previously unknown fungal disease, chytridiomycosis (Berger et al. 1998), and ranavirus infections (Cunningham et al. 1996, Cunningham 2001) have been reported as the cause of amphibian mass mortality associated with population declines (Daszak et al. 1999). These emerging infectious diseases (EIDs) are part of a growing cohort of wildlife EIDs that threaten biodiversity globally (Daszak et al. 2000). The current investigation was conducted to assess the potential role of infectious disease in a recent decline of *Rana lessonae* at Solomeo, Umbria, Italy.

The *Rana esculenta* complex is a group in which clonal reproduction has arisen. *R. esculenta* (*E*) are natural hybrids between *R. ridibunda* (*R*) and *R. lessonae* (*L*) (Berger 1967, Günther 1973, Graf & Polls Pelaz 1989). Both sexes of the hybrids reproduce hemiclonally via a hybridogenetic gametogenesis (Schultz 1969). In the germ line of these hybrids, the *L* genome is excluded before meiosis, the remaining *R* genome

*Corresponding author.
Email: daszak@conservationmedicine.org

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undergoes a premeiotic or occasionally a prediplotene meiotic endoreduplication (Tunner & Heppich-Tunner 1991) and 2 apparently normal meiotic divisions resulting in functional, genetically identical haploid gametes that contain an unrecombined *R* genome. Hybridity in these lineages is restored each generation through fertilization of these gametes by gametes from syntopic *L*. The vast majority of *E* lineages coexist as sexual parasites with the host species *L*.

The *L-E* system population is widespread throughout Europe (Uzzell & Berger 1975). The proportion of parental species and hybrids in natural populations depends on the environment. It it hypothesized that this is because hybrids are more tolerant to environmental stress than parental species: *E*, in contrast to *L*, appears to produce metamorphs that are better adapted to hypoxic conditions and fungicides ordinarily used in agriculture (Tunner & Nopp 1979, Semlitsch & Reyer 1992, Fioramonti et al. 1997, Hotz et al. 1999). Because of this apparent dichotomy in susceptibility to environmental variables, the hybridogenetic system may represent an interesting model for examining the ecology of pathogens. A hybridogenetic *L-E* system is present on the Italian peninsula; it is formed by a *lessonae*-like parental species and its *esculenta*-like hybrid (Uzzell & Hotz 1979, Uzzell 1983, Günther & Plötner 1994). Previous research on the composition of the hybridogenetic system conducted in the Trasimeno Lake district in Central Italy suggests that the parental species may be threatened by environmental stressors (Bucci et al. 2000). It is of interest that in a restricted area (Solomeo), close to Trasimeno Lake, a frequency reduction has been observed in the parental species with respect to the hybrid form within the time span of 1998 to 2000. In this paper we report the preliminary results of pathological and parasitological investigations on this population. The enigmatic parasite *Dermocystidium ranae* is reported from both the parental species and the hybrid form. The incidence was significantly higher in the parental species. The ultrastructure of mature *Dermocystidium* cysts is described and its taxonomy discussed. It remains unknown if this parasite is a significant pathogen of green frogs, and the cause of decline in the parent population has yet to be deduced.

MATERIALS AND METHODS

Collections of specimens. Live, metamorphosed green frogs of similar size were collected from ponds and streams within cultivated fields in the Solomeo area, near Trasimeno Lake in Central Italy (Umbria) in the years 1999 to 2000. The frogs were released into their habitat after clinical examination, ventral and toe skin biopsy (for pathological examination) and phalanx removal (for species determination). All procedures were carried out under general anaesthesia.

Host species determination. Species determination was carried out using the centromeric satellite deoxyribonucleic acid (DNA) marker RrS1, to reliably distinguish between non hybrid and hybrid frogs in Southern blot and fluorescent *in situ* hybridization (Ragghianti et al. 1995 and Bucci et al. 2000 provided technical details).

Statistical analysis. The Pearson chi-squared test for contingency (Kirkwood 1988) was used to analyze parasitism incidence data (positive vs negative).

Pathological examinations. Frogs were examined using a stereomicroscope and photographed. Ventral and toe skin in which gross evidence of parasitism was detected by the presence of U-shaped swellings were processed for further examination using light and electron microscopy.

For light microscopy, the specimens were fixed in 4% paraformaldehyde at 4°C, dehydrated in graded ethanols, embedded in paraffin wax, sectioned at 4 µm and stained with Haematoxylin and Eosin.

For transmission electron microscopy (TEM), the samples were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanols and embedded in Epon-Araldite. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a Philips 400 TEM at 60 kV.

RESULTS

U-shaped swellings (Fig. 1) were detected in frogs examined during each year of the research. Over the course of this study, the incidence of infection was significantly higher in the parental species compared to the hybrid form ($c_1^2 = 11.61$, $p < 0.001$) (Table 1). Parasite stages, comprising large clusters of encysted spores similar to those reported previously as *Dermocystidium ranae*, were recorded in all U-shaped swellings examined histologically (n = 25). These cysts were located in the stratum spongiosum of the dermis (Figs. 2 to 4). The cysts provoked a mild inflammatory (predominantly mononuclear) cell response (Fig. 3). Ultrastructural examination (Figs. 5 to 9) showed that mature cysts of *Dermocystidium* sp. contain numerous spores, each with multiple osmiophilic granules, a single membrane-bound nucleus, prominent nucleolus and a thick spore wall (Fig. 5). The cyst wall was composed of an osmiopilic shell attached to the connective tissue of the host, with a thickened inner wall comprised of an array of fibres packed closely together (Fig. 6). The spore wall was similarly complex, with a thickened outer layer of osmiophilic fibrous material, an intermediate bilayered

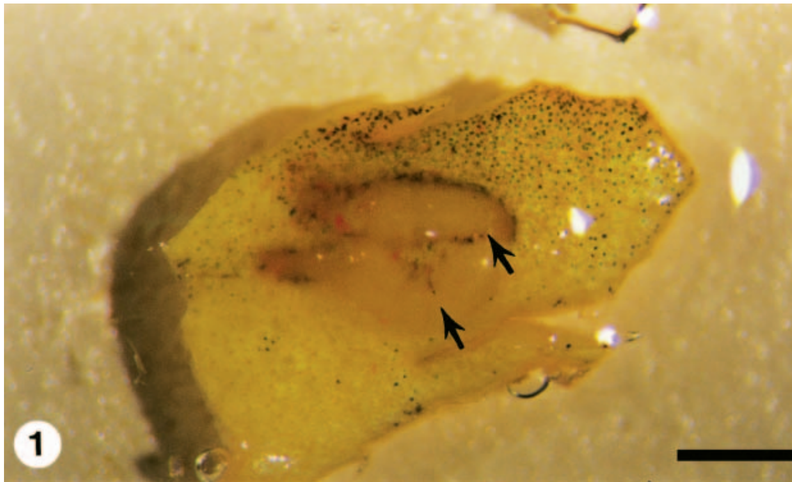


Fig. 1. *Rana lessonae*. Stereoscope photomicrograph of a typical U-shaped swelling on the ventral surface of an individual of the parental species *R. lessonae*. Arrows: the 2 sides of the U-shaped swelling. Scale bar = 5 mm

membrane and an inner irregular (as if furrowed) layer adjacent to the spore cytoplasm (Fig. 7). In many cases, spores appeared to be subspherical and closely packed, suggesting ability to conform in shape (Fig. 8). Mitochondria were not observed in any of the spores examined. Mature spores contained multiple electron-dense inclusion bodies and dense aggregations of ribosomes (Figs. 8 & 9). Despite examination of over 500 sections of mature spores, no evidence of flagella, an apical complex, or polar tubules was found.

DISCUSSION

Identity and clinical significance of the parasite

The current study reports parasitism by a *Dermocystidium* sp. in a population of parental and hybrid green frogs in a small area of Central Italy where, in 1999, a 50% reduction of the parental species occurred. *Dermocystidium* infections have been previously reported in other European anurans: in *Alytes obstetricans* (Pérez 1913), in *Rana temporaria* from Switzerland (Guyénot & Naville 1922), in *R. esculenta* from France (Remy 1931), and in *R. temporaria* in Czechoslovakia (Brož & Přívora 1951). The type species, *D. pusula*, has been reported from *Triturus cristatus*, *T. marmoratus* and *T. palmatus* (Pérez 1913, Poisson 1937). Yet other dermocystidians have been described from fish species, some of which are pathogenic (Cervinka et al. 1974, Olson et al. 1991, Landsberg & Paperna 1992, Wildgoose 1995).

The parasite reported in the present paper is identical grossly, histopathologically and developmentally to *Dermocystidium ranae*, reported from *Rana temporaria* by Guyénot & Naville (1922). Brož & Přívora (1951) also found *D. ranae* in *R. temporaria*, but reported spores with single-inclusion bodies, rather than the multiple granules reported from spores in the current paper. However, this is probably not a relevant character, since drawings by Guyénot & Naville (1922) show that both single and multiple bodies occur and Brož & Přívora (1951) supplied no photomicrographs or drawings to support their findings.

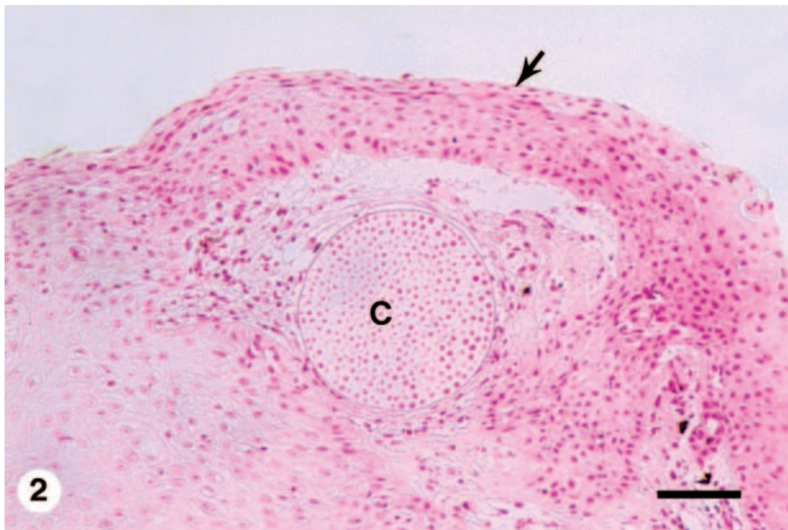
The pathogenic potential of *Dermocystidium ranae* remains uncertain. Brož & Přívora (1951) reported 5% incidence of *D. ranae* cysts in *Rana temporaria* from Czechoslovakia, supporting previous suggestions that *Dermocystidium* spp. are relatively benign parasites of amphibians (Guyénot & Naville 1922). Although the incidence of parasitism found in the current study is higher than that reported by Brož & Přívora (1951), we found no evidence that infection causes mortality or morbidity in green frogs. There was a significantly higher incidence of parasitism in *R. lessonae* (which declined by >50%) than in *R. esculenta* (stable population), but such data are insufficient for deducing the impact of infectious diseases on populations (McCallum & Dobson 1995). In the absence of transmission experiments and systemic post mortem examinations of wild-collected carcasses, the ecological significance of *Dermocystidium* sp. to amphibians remains unknown. The cause of the recent 50% reduction of the parental form has yet to be elucidated.

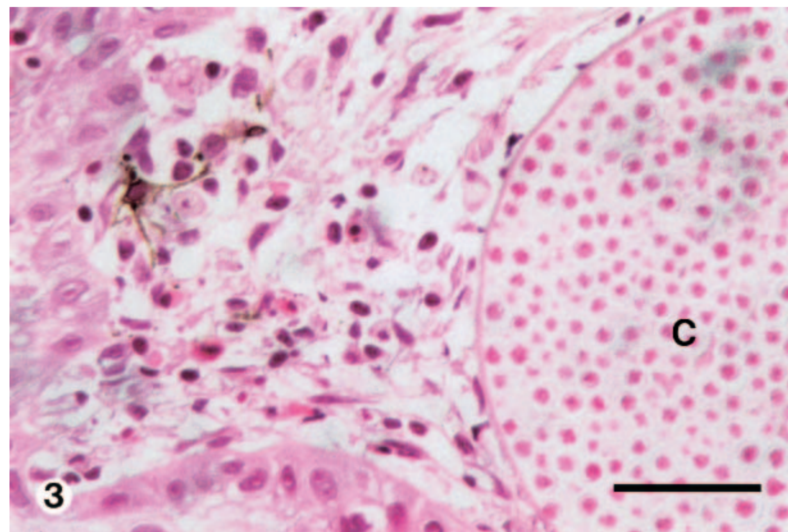
Table 1. Incidence of *Amphibiocystidium ranae* n. comb. cysts in nonhybrids and hybrids of a *Rana esculenta* complex population

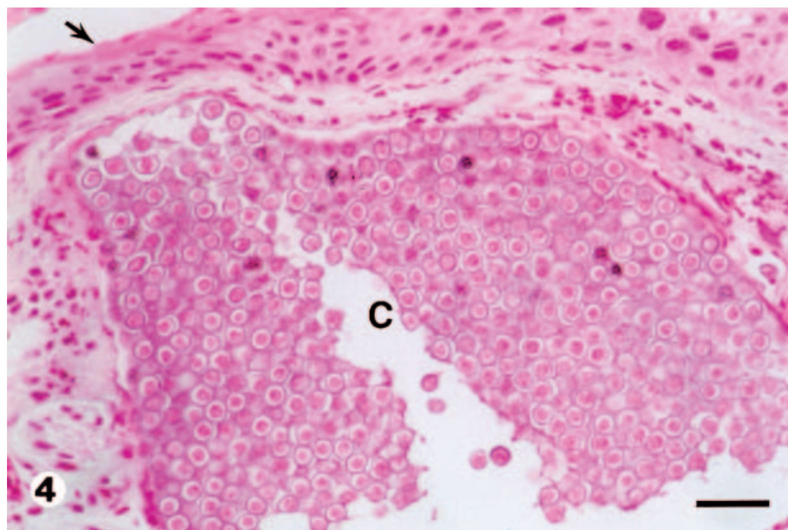
Frog taxon	Year collected	No. infected	Total examined	% incidence
R. lessonae	1999	10	22	45.5
R. esculenta	1999	3	21	14.3
R. lessonae	2000	11	21	52.4
R. esculenta	2000	5	29	17.2

Taxonomic revision

The original description of the genus *Dermocystis* (later renamed *Dermocystidium*) was published almost 100 yr ago (Pérez 1907). Since then, many of the diagnostic characters have been invalidated (see Table 2



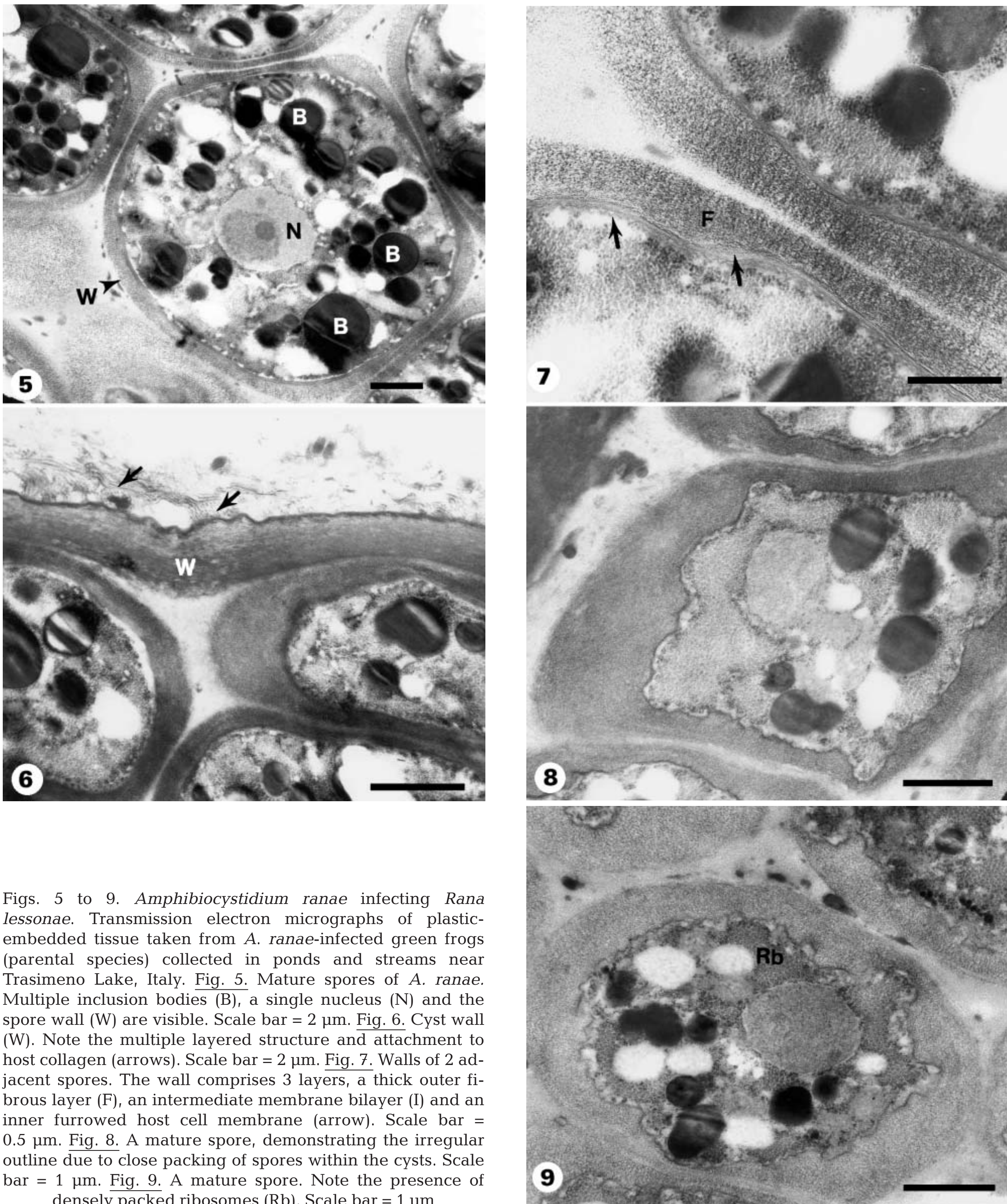




Figs. 2 to 4. *Rana esculenta*. Photomicrographs of H&E-stained paraffin sections of the hind feet of the hybrid form *R. esculenta* from ponds and streams near Trasimeno Lake, Italy. Fig. 2. A parasite cyst (c) within the stratum spongiosum of the dermis containing numerous spores. Note the almost complete absence of an inflammatory, or other tissue, reaction. Arrow: superficial epidermis. Scale bar = 100 µm. Fig. 3. Higher magnification of the parasite cyst (c) in Fig. 2. A slight infiltrate of mixed inflammatory cells is visible to the left of the cyst. Scale bar = 50 µm. Fig. 4. A parasite cyst (c), similar to that shown in Fig. 2, but one in which the cyst wall is less obvious and with protrusion of the cyst towards the skin surface (arrow). Scale bar = 50 µm

and discussion below) and techniques such as TEM and DNA sequencing have uncovered new taxonomically useful characters. Table 2 lists key taxonomic criteria for all amphibian and 3 fish *Dermocystidium* spp., and 2 other closely related genera from amphibians. These characters are not adequate to distinguish between species, and it is clear that the validity of all 3 genera in amphibians and their relationship to fish dermocystidians is in question. We propose the following taxonomic changes to address these problems:

(1) Synonymy of *Dermosporidium multigranulare* and *Dermocystidium ranae*. Brož & Kulda (1954) separated *D. multigranulare* from *D. ranae* by the spherical cyst shape, presence of septa around single spores in the former and differences in spore size. The first character is invalidated by the authors' description of *D. multigranulare* cysts comprising 'an elongated cylinder bent in the form of a U'. The second character is invalidated because Guyénot & Naville (1922) also reported septa around single spores of *D. ranae*. The authors use spore size as a character but do not state whether measurements were made of live or fixed spores, or on spores within fixed and processed tissue. They also do not give ranges. Wide variations in spore size for other parasites in these genera suggest that overlap is very likely between *D. multigranulare* and *D. ranae*.



Figs. 5 to 9. *Amphibiocystidium ranae* infecting *Rana lessonae*. Transmission electron micrographs of plastic-embedded tissue taken from *A. ranae*-infected green frogs (parental species) collected in ponds and streams near Trasimeno Lake, Italy. Fig. 5. Mature spores of *A. ranae.* Multiple inclusion bodies (B), a single nucleus (N) and the spore wall (W) are visible. Scale bar = 2 µm. Fig. 6. Cyst wall (W). Note the multiple layered structure and attachment to host collagen (arrows). Scale bar = 2 µm. Fig. 7. Walls of 2 adjacent spores. The wall comprises 3 layers, a thick outer fibrous layer (F), an intermediate membrane bilayer (I) and an inner furrowed host cell membrane (arrow). Scale bar = 0.5 µm. Fig. 8. A mature spore, demonstrating the irregular outline due to close packing of spores within the cysts. Scale bar = 1 µm. Fig. 9. A mature spore. Note the presence of densely packed ribosomes (Rb). Scale bar = 1 µm

(2) Synonymy of the genera *Dermomycoides*, *Dermosporidium* and amphibian *Dermocystidium* parasites and designation of *Amphibiocystidium* n. gen. Previous workers (Guyénot & Naville 1922, Brož & Přívora 1951, Brož & Kulda 1954) distinguished cysts of *Dermocystidium* spp. and *Dermosporidium* spp. primarily by the U-shaped cysts and presence of septa around single spores in the former. Table 2 reveals

Table 2. Key taxonomic characters of amphibian *Dermocystidium*, *Dermosporidium* and *Dermomycoides* spp. For all species, the site of development is the skin, except those from fish for which the site is listed. ?: some discrepancies in the authors' comments or interpretation; –: unknown

Species	Source	Host	TEM study	Site of development	Cyst size/shape	Septa between groups of spores	Single or multiple granules	Spore size (µm)	Cytoplasm crescent-shaped[c]	Zoospores
Amphibian *Dermocystidium* spp.										
D. pusula	Pérez (1907)	*Triturus marmoratus*	No		Spherical		Single	8–10	Yes	No
	Pérez (1913)	*T. cristatus*, *Alytes obstetricans*								
D. ranae	Guyénot & Naville (1922)	*Rana temporaria* *R. esculenta* *Alytes obstetricans*	No		U-shaped	No, around single spores	Both	7–9	Yes	No
D. ranae	Present paper	*R. esculenta* complex	Yes		U-shaped	No	Multiple		No	No
Amphibian *Dermosporidium* spp.										
D. hyalarum (*hylae*)	Carini (1940)	*Hyla rubra*	No		Spherical?	No?	Multiple	8–10	–	No
D. granulosum	Brož & Přívora (1951)	*R. temporaria*	No		Spherical	Yes?	Multiple	10	–	No
D. multigranulare	Brož & Kulda (1954)	*R. esculenta*	No		Spherical to U-shaped	No	Multiple	18	–	No
D. penneri	Jay & Pohley (1981)[b]	*Bufo americanus*	No		Spherical	Yes	Multiple	10–12	–	No
Amphibian *Dermomycoides* spp.										
D. beccarii	Granata (1919)		No							
D. armoriacus	Poisson (1937)	*Triturus palmatus*	No		Spherical	–	–		–	Yes
Selected fish *Dermocystidium* spp.										
D. salmonis[a]	Davis (1947)	*Oncorhynchus tshawtscha*	Yes	Gill epithelium	Small, spherical		Single	5–8	Yes	Yes
D. cyprini	Červinka et al. (1974)	*Cyprinus carpio*	No	Gill epithelium	Elongate	Yes	Single		Yes	No
D. macrophagei[a]	Moer et al. (1987)	*Salmo gairdneri*	Yes	Kidney macrophages	Cysts absent	No	Multiple	2–8	Yes	No

[a]Spore structure confirmed by TEM
[b]See also Green & Kagarise Sherman (2001) in *Bufo canorus*. A disseminated infection of *D. penneri* was reported. Identification was by histological analysis and light microscopy
[c]This has been readily observed for a number of species and essentially represents the presence of a single inclusion body (a character sometimes missed by authors) that pushes cytoplasm to the side

inconsistencies and incomplete data for both criteria in both genera. Other criteria used are also inadequate: although the depth of development varies slightly, cysts of all species develop in the connective tissue of the superficial dermis just below the basement membrane of the epidermis; slight variations exist in the pathological changes around cysts, but these may partly be due to observations of cysts at different stages of development. Neither criteria are widely used as generic taxonomic characters in other parasitic groups, and the latter is essentially a response to infection. In his description of the third genus in this group, *Dermomycoides*, Granata (1919) published 'preliminary results' of his observations, pending 'research on living material and possibly attempting some cultures and artificial infections' – work that was unfortunately not completed. Granata (1919) used presence of septa around single spores, site of development and histopathological observation of the host reaction to cyst development as distinguishing characters. For the reasons outlined earlier, these are insufficient for generic status. Furthermore, cysts of *Dermomycoides* spp., *Dermosporidium* spp. and *Dermocystidium* spp. are macroscopic, spherical in transverse section, located just below the epidermis and therefore indistinguishable histopathologically (Granata 1919, Poisson 1937). The only exception to this is the recent report of a disseminated *Dermosporidium penneri* infection in a single speciment of *Bufo canorus* (Green & Kagarise Sherman 2001). Macroscopic dermal cysts consistent with *D. penneri* were reported along with the presence of cells identical to *D. penneri* spores in visceral organs. Because this is the only report of a systemic infection by a *Dermomycoides* spp., *Dermosporidium* spp. or *Dermocystidium* spp., we suggest that it does not have taxonomic significance, however further work (TEM, DNA sequencing) should be conducted on this specimen to further confirm the parasite's identity.

The classification of *Dermomycoides armoriacus* is blurred by the description of 3 types of cyst: large 'primary' cysts containing spherical spores, small 'secondary' cysts containing flagellated zoospores, and occasionally observed 'durable' cysts (Poisson 1937). In our opinion, it is possible that the 'durable' cysts are cysts that failed to mature and became encapsulated. Poisson (1937) suggested that the contents of 'secondary' cysts denote affinity to the Chytridiomycota (chytridiales). It is possible that these structures represent a stage in the life cycle of amphibian *Dermosporidium*, *Dermocystidium* or *Dermomycoides* that has been missed by previous workers. It is equally likely that the authors were observing a dual infection with a zoosporic fungus (possibly the first description of chytridiomycosis). We propose that these secondary cysts should not be used in generic considerations until further specimens are available. In conclusion, we propose that all 3 genera are *nomina dubia* when applied to amphibian host species and are synonymous.

Amphibian parasites of all 3 genera are significantly different in structure and development to fish *Dermocystidium* spp., therefore designating them as *Dermocystidium* is not appropriate. Fish dermocystidians occur in a range of tissues and their morphology varies from species that lack a cyst stage through those that form microscopic cysts to those with macroscopic cysts. Fish *Dermocystidium* spp. have been repeatedly recovered and characterized, and some have a zoosporic phase to their life cycle that is well established. Published information exists on the ultrastructure, DNA sequence phylogeny and life cycles of fish dermocystidians, whereas the amphibian parasites are very poorly known, with only 1 TEM report (present paper), no sequence data, no full life cycles elucidated, and transmission experiments carried out on none. Following the arguments above, we consider designation of a new genus for the current 3 amphibian genera the most logical and parsimonious option for this group of organisms. The genus *Dermocystidium* was described from an amphibian and therefore would normally be retained for this group of amphibian parasites. However, the *Dermocystidium* spp. parasitic in fish have been the subject of far more intense scientific interest over the past 50 yr than their amphibian counterparts. To avoid reclassification of the fish parasites, we believe that *Dermocystidium* should be retained for fish *Dermocystidium* spp. The International Commission for Zoological Nomenclature (ICZN) allows for such cases, under article 23.9.3, to avoid confusion or instability (International Commission on Zoological Nomenclature 1999). We have formally requested planary action from the ICZN to designate *Dermocystidium salmonis* Davis, 1947 (the most well-characterized *Dermocystidium* sp. from fish) as the type for this genus (P. Daszak & A. A. Cunningham unpubl.). Fish *Dermocystidium* spp. can easily be distinguished from the amphibian parasites by their occurrence in piscine hosts, their ultrastructural differences (see below) and their life cycle details.

Characters originally used to define *Dermocystis* are insufficient for the purposes of designating the amphibian parasites, as are those for *Dermosporidium*. Using these genera to group all amphibian parasites named in Table 2 would likely lead to confusion by implying that the characters used to define these genera were valid. Herr et al. (1999) commented that *Dermosporidium hyalarum* (Carini 1940), often mis-named *D. hylae*, and *D. granulosum* may be synonymous with *Rhinosporidium seeberi*, partly because Carini (1940) originally named *D. hyalarum* as a *Rhinosporidium* sp.

However, Herr et al. (1999) made this comment based on some histological similarities between the original descriptions of the amphibian parasites and data on *R. seeberi*, and their parasitism of terrestrial vertebrates. No DNA sequence data, nor detailed life history nor detailed morphological information (high-quality photomicrographs, or electron micrographs) for the amphibian species have been published that support such an assumption.

We propose that the amphibian parasites listed in Table 2 be grouped in a new genus, *Amphibiocystidium* n. gen. (Table 3). Type specimens (including resin-embedded blocks for electron microscopy) of *Amphibiocystdium ranae* n. gen. n. comb. have been deposited at the Zoological Society of London's Pathology Museum Archive, accession number H50/99. Paratypes are H49/99, H41/01 and H42/01. We therefore believe that the most parsimonious taxonomy is that *Amphibiocystidium* n. gen. n. comb. is a sister taxon to *Dermocystidium* and *Rhinosporidium*. The taxonomy of these groups is unlikely to remain static; however, placing *Amphibiocystidium* within *Rhinosporidium* or *Dermocystidium* does not represent an accurate synthesis of our current knowledge (including data within this paper).

For many groups of organisms, the taxonomic changes proposed above would be considered significant. However, it is important to note that in the almost 100 yr since the first reports of these genera, a number of authors have proposed synonymy between them or other genera, and a number of species have been reclassified. Furthermore, no full life cycles have been described for any of the amphibian parasites, and neither sequencing nor comparative ultrastructural studies have been performed. At best, these parasites were described from small numbers of specimens collected by different authors decades apart and during a time when systematics of the basal fungal-protistan lineages was poorly known. Some were described from preliminary results of fixed samples from a single-host specimen, or possible dual infections. Further studies (e.g. sequencing, life cycle, transmission experiments, TEM) may allow further insights into their taxonomy, but we believe our revisions represent the most efficient and useful classification at present.

Table 3. Taxonomic designation of *Amphibiocystidium* n. gen.

***Amphibiocystidium* n. gen.**
Type species: *Amphibiocystidium ranae* n. comb.[a]
Type host: *Rana esculenta* complex, a hybridogenetic system of green frogs including parental species (*R. lessonae*) and clonal hybrids (*R. esculenta*).
Type locality: Solomeo, Umbria, Central Italy
Description: Parasites infecting the dermis of amphibian hosts. Macroscopic, spherical-subspherical or U-shaped cysts containing numerous spherical-subpspherical spores 7 to 12 µm in diameter. Hyphae not present, flagellated zoospores not present, polar tubes not present, apical complex not present.
Species designated to this genus (and authorities): *Dermocystidium pusula* Pérez, 1907 *Dermocystidium ranae* Guyenot & Naville, 1922 *Dermosporidium hylae* Carini, 1940 *Dermosporidium granulosum* Brož & Přívora, 1951 *Dermosporidium multigranulare* Brož & Kulda, 1954 *Dermosporidium penneri* Jay & Pohley, 1981 *Dermomycoides beccarii* Granata, 1919 *Dermomycoides armoriacus* Poisson, 1937
[a]Type specimens of *Amphibiocystdium ranae* n. gen. n. comb. have been deposited at the Zoological Society of London's Pathology Museum Archive, accession number H50/99. Paratypes are H49/99, H41/01 and H42/01

Phylogenetic position of *Amphibiocystidium* n. gen.

The phylogenetic position of fish *Dermocystidium* spp. has recently been reassessed and the genus designated to the Dermocystidium–Rosette agent–Ichthyophonous–Psorospermium ('DRIPs') clade of aquatic parasites that infect fish, amphibians and humans (Ragan et al. 1996, Herr et al. 1999, Fredricks et al. 2000). More recent taxonomic review places fish *Dermocystidium* and other DRIPs within the Kingdom Protozoa, Phylum Neomonada, Class Mesomycetozoea (Mendoza et al. 2002). This class is in an interesting phylogenetic position near the animal-fungal divergence and contains genera with previously uncertain taxonomic designation. Thus, we propose that the genus *Amphibiocystidium* probably represents a sister clade to *Dermocystidium* and *Rhinosporidium* within the Mesomycetozoea.

The present study represents the first ultrastructural analysis of a species previously assigned to the genus *Dermocystidium* in amphibians and confirms their Eukaryotic nature. Despite suboptimal infiltration of cysts and the examination of only mature stages, a preliminary comparison between *Amphibiocystidium ranae* n. sp. and *Dermocystidium* species from fish hosts can be made. Firstly, no zoospores were found in the former. Olsen et al. (1991) demonstrated that flagellated zoospores of *D. salmonis* develop from mature spores with single inclusion bodies after 15 d incubation in fresh water at 4°C. Developing zoospores were observed within spores only after removal from infected gills and following incubation in fresh water. If this were the case for *Amphibiocystidium* n. gen., we would expect to see flagella or basal bodies inside the spores, both of which were absent. Thus, although we cannot confirm that *Amphibiocystidium* n. gen. is not a flagellated zoosporic group of organisms until further

work on excysted spores has been conducted, it appears unlikely. The ultrastructure of the spores described in the current study differs from that of any *Dermocystidium* sp. of fish described by TEM. Firstly, no fish spp. have similar multiple dense bodies. *D. macrophagei* contains multiple small granules only during early spore maturation (Moer et al. 1987), whereas Olsen et al. (1991) report only single inclusion bodies from *D. salmonis*. Dense aggregations of ribosomes are present within *A. ranae* n. comb. spores whereas spores of fish *Dermocystidium* spp. contain ribosomes disseminated throughout the cytoplasm. Our ultrastructural findings therefore support this genus' separate status to fish *Dermocystidium*.

None of the structures observed in the current study suggest that *Amphibiocystidium* n. gen. belongs to a Microsporidian or Apicomplexan lineage, and it is clear that there is a close relationship to fish *Dermocystidium* spp. Until DNA sequence analysis, or ultrastructure of the *Amphibiocystidium* mitochondrion is reported, we would suggest that this group is a sister genus of fish *Dermocystidium* and likely another member of the DRIPs clade (Ragan et al. 1996).

CONCLUSIONS

Our paper reports the presence of an enigmatic parasite from an important amphibian hybridogenetic system. Future work may allow a more accurate assessment of the pathogenicity of this and related infections in the parental species and hybrid forms, and further our understanding of the ecology of parasites within this system. Our analysis of similar parasite species described from amphibians and fish support our conclusion that *Dermosporidium multigranulare* Brož & Kulda, 1954 is synonymous with *Dermocystidium ranae* Guyénot & Naville, 1922 and, due to lack of sufficient differences between genera and significant dissimilarities with fish *Dermocystidium* spp., the 3 amphibian genera are synonymous and should be designated to a new genus, *Amphibiocystidium* n. gen. *Amphibiocystidium* n. gen. holds an interesting phylogenetic position close to the fungal–animal divergence and to a number of important pathogens such as fish *Dermocystidium* species, *Batrachochytrium dendrobatidis*, the causative agent of amphibian chytridiomycosis (Longcore et al. 1999) and *Rhinosporidium sebeeri*, the agent of a granulomatous disease of humans and animals (Herr et al. 1999, Fredricks at al. 2000). We believe that future work on the taxonomy, phylogeny, life cycles and pathology of these parasites will therefore be of significance to the wider field of infectious disease biology.

Acknowledgements. We are grateful to Professor S. J. Ball (Kingston University) for useful discussions on taxonomy, to Mr. Carlo Corvaja for the translation of Italian papers, and to H. Hotz (University of Zurich), G.-D. Guex (University of Zurich) and G. Mancino (University of Pisa) for their helpful comments on the green frog complex. This work was supported by core funding for the Consortium for Conservation Medicine from the V. Kann Rasmussen Foundation, by grants from University of Perugia, Italy, and by a National Science Foundation IRCEB grant (IBN #9977063).

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Editorial responsibility: Peernel Zwart, Utrecht, The Netherlands

Submitted: March 20, 2002; Accepted: April 10, 2003
Proofs received from author(s): July 18, 2003